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(54) **Recombinant Feline herpesvirus vaccine.**

(57) The present invention is concerned with a Feline herpesvirus (FHV) mutant comprising a heterologous gene introduced into an insertion-region of the FHV genome.

The invention also relates to a vector vaccine comprising such an FHV mutant which expresses a heterologous polypeptide derived from a feline pathogen and induces an adequate immune response in an inoculated host against both FHV and the feline pathogen.

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The present invention is concerned with a feline herpesvirus (FHV) mutant comprising a mutation in a region of the FHV genome, a nucleic acid sequence comprising an FHV insertion-region, a nucleic acid sequence comprising a heterologous nucleic acid sequence flanked by DNA derived from the insertion-region, a recombinant DNA molecule comprising such nucleic acid sequences, a cell culture infected with an FHV mutant, as well as vaccine comprising the FHV mutant.

One of the major clinical problems in diseases of Felidae is associated with respiratory tract infections. The great majority of these cases are caused by either feline herpesvirus 1 (FHV) or feline calicivirus.

FHV is the causative agent of feline viral rhinotracheitis in cats. In kittens, FHV infection can generalize resulting in mortality rates of up to 50%. The disease is common and is found world-wide and is characterized by sneezing, depression, and ocular and nasal discharge.

The FHV is a member of the family Herpesviridae, subfamily α -herpesvirus. The genome is about 126 kbp in length and is composed of a unique long (U_L) region of about 99 kbp and a short region of 27 kbp comprising an unique short (U_S) region of about 9 kbp flanked by inverted repeats of about 8 kbp (Grail et al., Arch. Virol. 116, 209-220, 1991).

Because of the prevalence and seriousness of FHV infection feline viral rhinotracheitis vaccines comprising modified live or killed FHV have been developed and have resulted in a successful reduction of the incidence of the disease.

In addition to FHV infection, cats are also susceptible to infection by various other pathogens, such as feline leukemia virus (FeLV), feline calicivirus, feline immunodeficiency virus (FIV), feline coronavirus and feline Chlamydia. At present, in general, cats can be protected against infection by these pathogenic micro-organisms with live or inactivated vaccines or by vaccines derived from subunits of the relevant pathogens.

However, these types of vaccines may suffer from a number of drawbacks. Using attenuated live vaccines always involves the risk of inoculating animals with inadequately attenuated pathogenic micro-organisms. In addition the attenuated pathogens may revert to a virulent state resulting in disease of the inoculated animals and the possible spread of the pathogen to other animals.

Inactivated vaccines generally induce only a low level of immunity, requiring repeated immunizations. Furthermore, the neutralization inducing antigenic determinants of the pathogens may become altered by the inactivation treatment, decreasing the protective potency of the vaccine.

Moreover, a problem with combined live viral vaccines is the mutual influence of the antigenic components resulting in a decrease of the potency of one or more of the constituting components.

A recombinant or naturally derived subunit vaccine also displays a number of disadvantages. First, a polypeptide subunit presented to the immune system as a non-replicating structure often does not elicit long-lasting immunity requiring also the presence of an adjuvant. Secondly, a presentation as a replicating structure can elicit immunity more efficiently than can a presentation as a subunit structure.

Furthermore, with currently administered live attenuated or inactivated FHV vaccines it is not possible to determine whether a specific animal is a carrier of an FHV field virus or whether the animal was vaccinated. Hence, it is important to be able to identify animals vaccinated with an FHV vaccine or infected with a field virus so as to be able to take appropriate measures to reduce spreading of a virulent field virus.

It is an object of the present invention to provide an FHV mutant which can be used not only for the preparation of a vaccine against feline viral rhinotracheitis but also against other infectious diseases of Felidae, which obviates any potential risk associated with the use of a live attenuated pathogen as a vaccine, which stimulates both the humoral and cellular immune system in a potent way without the explicit need of an adjuvant and which offers the possibility of a multivalent vaccine without the risk of adverse mutual interference of different antigenic components.

An other object of the present invention is to provide an FHV vaccine virus which is distinguishable from any field strain or any other FHV vaccine virus.

The present invention provides an FHV mutant comprising a mutation in the FHV genome in a region defined by the DNA sequence of the open reading frame encoding a polypeptide shown in SEQ ID NO: 2 and flanking intergenic sequences thereof.

A mutation is understood to be a change of the genetic information in the above-mentioned region with respect to the genetic information present in this region of the genome of naturally occurring FHV. The mutation is, for example, a nucleic acid substitution, deletion, insertion or inversion, or a combination thereof resulting in an FHV mutant which fails to produce any antigenic or functional polypeptide shown in SEQ ID NO: 2.

Preferably, the mutation introduced into the defined region of the FHV-genome is a deletion or insertion.

In particular the present invention provides a recombinant FHV mutant characterized in that it comprises a heterologous nucleic acid sequence, said nucleic acid sequence being introduced in the region of the FHV genome defined by the DNA sequence of the open reading frame (ORF) encoding a polypeptide

shown in SEQ ID NO: 2 and flanking intergenic sequences thereof.

The FHV mutant according to the present invention can be derived from any FHV strain, e.g. strain G2620 (commercially available from Intervet International B.V., the Netherlands), C-27 (ATCC VR-636), FVRm (ATCC VR-814), FVRm vaccine (ATCC VR-815) or F2.

5 The term "recombinant FHV mutant" as used herein denotes infective virus which has been genetically modified by incorporation into the virus genome of a heterologous nucleic acid sequence, i.e. DNA which comprises a nucleic acid sequence not identical to the nucleic acid sequence of a gene naturally present in FHV.

On infection of a cell by the recombinant FHV mutant, it may express the heterologous gene in the form of a heterologous polypeptide.

The term "polypeptide" refers to a molecular chain of amino acids, does not refer to a specific length of the product and if required can be modified in vivo or in vitro, for example by glycosylation, amidation, carboxylation or phosphorylation; thus inter alia peptides, oligopeptides and proteins are included within the definition of polypeptide.

15 The prerequisite for a useful (recombinant) FHV mutant is that the mutation such as an inserted heterologous nucleic acid sequence is incorporated in a permissive position or region of the genomic FHV sequence, i.e. a position or region which can be used for the incorporation of the mutation without disrupting essential functions of FHV such as those necessary for infection or replication. In the case of the insertion of a heterologous nucleic acid sequence such a region is called an insertion-region.

20 Until now little is known about the localization of genes on the FHV genome. Rota et al. (Virology 154, 168-179, 1986) and Grail et al. (Arch. Virol. 116, 209-220, 1991) disclosed physical maps of the FHV genome. Nunberg et al. (J. Virology 63, 3240-3249, 1989) and Cole et al. (J. Virology 64, 4930-4938, 1990) identified the thymidine kinase (TK) gene and mapped this gene in the Sall-A restriction fragment (Rota et al., supra) of the FHV genome. Subsequently, several recombinant FHV strains were constructed in which

25 FeLV env and gag genes have been inserted within the FHV TK gene. The (insertion-) region referred to in the present invention has not been identified previously within the FHV genome. Surprisingly, it has been found that a mutation such as the incorporation of heterologous DNA is allowable in this region without disrupting essential functions of the FHV.

Even more unexpected, it has been found that the introduction of a mutation into the region defined above significantly reduces the virulence of the live FHV mutant without affecting the protective properties of the FHV mutant. This finding has offered the possibility to obtain an attenuated FHV mutant, e.g. by introducing a deletion or insertion into the region defined above, which mutant can be administered safely to the animals to be vaccinated in a live form, even via the oro-nasal route.

30 The (insertion-) region used to introduce a mutation such as the insertion of a heterologous DNA sequence in order to prepare a FHV mutant according to the invention is located within a 10.9 kb restriction fragment generated by partial digestion of genomic FHV DNA with the enzyme Sau3A.

Said fragment is analyzed in detail by restriction enzyme mapping and essentially corresponds to a region within the U_L segment of the viral genome between map unit 0.08 and 0.17 on the map of Grail et al. (1991, supra).

40 The (insertion-)region disclosed herein is located within a Sall fragment and comprises the DNA sequence encoding a polypeptide of 193 amino acids as shown in SEQ ID NO: 2 as well as its upstream and downstream flanking intergenic sequences. These upstream and downstream flanking intergenic sequence do not form part of an ORF or protein encoding DNA sequence, or do not comprise sequences regulating the replication of the virus. Said flanking sequences extend in the upstream and downstream

45 direction up to the start or end of the nearest open reading frame. Preferably, the upstream and downstream flanking intergenic sequences are about 252 and 173 bp in length, respectively.

It is a preferred object of the present invention to provide a (recombinant) FHV mutant that contains a mutation, such as a heterologous nucleic acid sequence into the (insertion-)region essentially defined by the DNA sequence shown in SEQ ID NO: 1.

55 In particular, a mutation such as a heterologous DNA sequence is incorporated into the FHV DNA sequence encoding the 193 amino acid long polypeptide defined by the amino acid sequence shown in SEQ ID NO: 2, and more specifically by its DNA sequence corresponding to the nucleotide position 253-834 shown in SEQ ID NO: 1, the unique BglII restriction site at nucleotide position 576 being the most favourable site for insertion of the heterologous DNA.

It will be understood that for the DNA sequence of the FHV genome, natural variations can exist between individual FHV viruses. These variations may result in deletions, substitutions, insertions, inversions or additions of one or more nucleotides. These FHV variants may encode a corresponding ORF that differs

from the ORF disclosed herein. The DNA sequence encoding such variant ORFs can be located by several methods, including hybridization with the DNA sequence provided in SEQ ID NO: 1, or comparison of the physical map to locate analogous regions encoding said ORF. Therefore, the present invention provides an (insertion-)region obtainable from any strain of FHV.

Moreover, the potential exists to use genetic engineering technology to bring about above-mentioned variations resulting in a DNA sequence related to the DNA sequence of the (insertion-)region defined above. It is clear that a (recombinant) FHV mutant comprising a mutation, such as an inserted heterologous gene incorporated into an (insertion-)region located within the FHV genome characterized by such a related DNA sequence is also included within the scope of the present invention.

Furthermore, as the (insertion-) region identified according to the present invention does not display essential functions, said region can be deleted partially or completely, whereafter a heterologous gene can be incorporated into said deletion if desired.

In summary, the (insertion-)region essentially defined above characterizes the localization of a region within the FHV genome which can be used to incorporate a heterologous nucleic acid sequence, if desired after deleting DNA sequences from this region, or can be used to introduce other mutations, in particular a deletion in said region.

The heterologous nucleic acid sequence to be incorporated into the FHV genome according to the present invention can be derived from any source, e.g. viral, prokaryotic, eukaryotic or synthetic. Said nucleic acid sequence can be derived from a pathogen, preferably a feline pathogen, which after insertion into the FHV genome can be applied to induce immunity against disease.

Preferably nucleic acid sequences encoding a polypeptide of feline leukemia virus, feline immunodeficiency virus, feline calicivirus, feline parvovirus, feline coronavirus and feline Chlamydia are contemplated for incorporation into the insertion-region of the FHV genome.

Furthermore, nucleic acid sequences encoding polypeptides for pharmaceutical or diagnostic applications, in particular immuno-modulators such as lymphokines, interferons or cytokines, may be incorporated into said insertion-region.

An essential requirement for the expression of the heterologous nucleic acid sequence in a recombinant FHV mutant is an adequate promotor operably linked to the heterologous nucleic acid sequence. It is obvious to those skilled in the art that the choice of a promotor extends to any eukaryotic, prokaryotic or viral promotor capable of directing gene transcription in cells infected by the recombinant FHV, e.g. promoters of the retroviral long terminal repeat (Gorman et al., Proc. Natl. Acad. Sci. USA 79, 6777-6781, 1982), the SV40 promotor (Mulligan and Berg, Science 209, 1422-1427, 1980) or the cytomegalovirus immediate early promotor (Schaffner et al., Cell 41, 521-530, 1985).

In case a deletion mutant according to the invention is desired, either partial or complete deletion of the region from the viral genome identified above can be achieved by the technique of in vivo homologous recombination.

First, a DNA fragment comprising part of the unique long sequence as defined in SEQ ID No.:1 and flanked by at least 100 nucleotides on either site, can be subcloned into a convenient plasmid vehicle.

The deletion to be introduced in the described region can be made in this plasmid by a restriction digest with one or more enzymes of which the sites are correctly positioned in or near the open reading frame. Recircularization of the remaining plasmid molecule would result in a derivative lacking at least part of the coding sequence present within the newly identified region. Alternatively, progressive deletions can be introduced either in one or two directions starting from within a restriction site present within the sequence of the open reading frame. Enzymes such as Ball or exonuclease III can be used for this purpose. Recircularized plasmid molecules are transformed into E.coli cells and individual colonies are analyzed by restriction mapping in order to determine the size of the deletion introduced into the specified region. An accurate positioning of the deletion can be obtained by sequence analysis. The plasmid containing a defined deletion can be cotransfected with FHV viral DNA into cultured feline cells. After in vivo recombination has occurred, the deletion will be introduced at the correct position within the described region of the viral genome. Recombinants among the viral progeny can be identified for example by means of 15 to 20 bases long synthetic oligomer which hybridizes specifically to the nucleotide sequence which is generated at the junction where the deletion originally was introduced.

The technique of in vivo homologous recombination can be used to introduce the heterologous nucleic acid sequence into the FHV genome. This is accomplished by first constructing a recombinant DNA molecule for recombination with FHV genomic DNA. Such a molecule may be derived from any suitable plasmid, cosmid or phage, plasmids being most preferred, and contains a heterologous nucleic acid sequence, if desired operably linked to a promotor. Said nucleic acid sequence and promotor are introduced into a fragment of genomic FHV DNA containing insertion-region sequences as defined herein

subcloned in the recombinant DNA molecule. The insertion-region sequences which flank the heterologous nucleic acid sequence should be of appropriate length, e.g. 50-3000 bp, as to allow in vivo homologous recombination with the viral FHV genome to occur. If desired, a construct can be made which contains two or more different heterologous nucleic acid sequences derived from the same or different pathogens said sequences being flanked by insertion-region sequences of FHV defined herein. Such a recombinant DNA molecule can be employed to produce recombinant FHV which expresses two or more different antigenic polypeptides to provide a multivalent vaccine.

Secondly, cells, e.g. feline kidney cells (CRFK) or feline embryo cells can be transfected with FHV DNA in the presence of the recombinant DNA molecule containing the heterologous nucleic acid sequence flanked by appropriate FHV sequences whereby recombination occurs between the insertion-region sequences in the recombinant DNA molecule and the insertion-region sequences in the FHV genome. Recombination can also be brought about by transfecting the infected cells with a nucleic acid sequence containing the heterologous nucleic acid sequence flanked by appropriate flanking insertion-region sequences without plasmid sequences. Recombinant viral progeny is thereafter produced in cell culture and can be selected for example genotypically or phenotypically, e.g. by hybridization, detecting enzyme activity encoded by a gene co-integrated along with the heterologous nucleic acid sequence or detecting the antigenic heterologous polypeptide expressed by the recombinant FHV immunologically. Recombinant virus can also be selected positively based on resistance to compounds such as neomycine, gentamycine or mycophenolic acid. The selected recombinant FHV can be cultured on a large scale in cell culture whereafter recombinant FHV containing material or heterologous polypeptides expressed by said FHV can be collected therefrom.

A live FHV mutant according to the present invention, and in particular a live recombinant FHV expressing one or more different heterologous polypeptides of specific pathogens, can be used to vaccinate animals, particularly domestic and non-domestic cats or canine species. Vaccination with such a live vector vaccine is preferably followed by replication of the recombinant FHV within the inoculated host, expressing in vivo the heterologous polypeptide along with the FHV polypeptides. The polypeptides expressed in the inoculated host will then elicit an immune response against both FHV and the specific pathogen. If the heterologous polypeptide derived from the specific pathogen can stimulate a protective immune response, then the animal inoculated with a recombinant FHV mutant according to the invention will be immune to subsequent infection by that pathogen as well as to infection by FHV. Thus, a heterologous nucleic acid sequence incorporated into the insertion-region of the FHV genome according to the invention may be continuously expressed in vivo, providing a solid, safe and longlasting immunity to a pathogen.

A recombinant FHV mutant according to the invention containing and expressing one or more different heterologous polypeptides can serve as a monovalent or multivalent vaccine.

For the preparation of a live vaccine the recombinant FHV mutant according to the present invention can be grown on a cell culture of feline origin. The viruses thus grown can be harvested by collecting the tissue cell culture fluids and/or cells. The live vaccine may be prepared in the form of a suspension or may be lyophilized.

In addition to an immunogenically effective amount of the recombinant FHV the vaccine may contain a pharmaceutically acceptable carrier or diluent.

Examples of pharmaceutically acceptable carriers or diluents useful in the present invention include stabilizers such as SPGA, carbohydrates (e.g. sorbitol, mannitol, starch, sucrose, glucose, dextran), proteins such as albumin or casein, protein containing agents such as bovine serum or skimmed milk and buffers (e.g. phosphate buffer).

Optionally, one or more compounds having adjuvant activity may be added to the vaccine. Suitable adjuvants are for example aluminium hydroxide, phosphate or oxide, oil-emulsions (e.g. of Bayol F^(R) or Marcol 52^(R)), saponins or vitamin-E solubilisate.

The useful dosage to be administered will vary depending on the age, weight and mode of administration. A suitable dosage can be for example about $10^{4.5}$ pfu/animal.

An FHV mutant according to the invention can also be used to prepare an inactivated vaccine.

For administration to animals, the FHV mutant according to the presentation can be given inter alia intranasally, intradermally, subcutaneously or intramuscularly.

It is a further object of the present invention to produce subunit vaccines, pharmaceutical and diagnostic preparations comprising a heterologous polypeptide expressed by a recombinant FHV mutant according to the invention. This can be achieved by culturing cells infected with said recombinant FHV under conditions that promote expression of the heterologous polypeptide. The heterologous polypeptide may then be purified with conventional techniques to a certain extent depending on its intended use and processed further into a preparation with immunizing, therapeutic or diagnostic activity.

The above described active immunization against specific pathogens will be applied as a protective treatment in healthy animals. It goes without saying that animals already infected with a specific pathogen can be treated with antiserum comprising antibodies evoked by a recombinant FHV mutant according to the invention comprising a heterologous gene derived from the specific pathogen encoding an antigenic polypeptide. Antiserum directed against a recombinant FHV according to the invention can be prepared by immunizing animals, for example cats, with an effective amount of said recombinant FHV in order to elicit an appropriate immune response. Thereafter the animals are bled and antiserum can be prepared.

EXAMPLE 1

Characterization of a new insertion region in the unique long sequence of the FHV genome.

- Preparation of FHV DNA and establishment of a genomic library in lambda vector EMBL4.

The vaccine strain of FHV-1 (commercially available as feline rhinotracheitis virus, strain G2620, from Intervet International B.V.; Holland) was grown on Crandell-Rees feline kidney (CRFK) cells (Crandell, R.A. et al., *In Vitro* 9, 176-185, 1973) in Glasgow's modified minimum essential medium supplemented with 2.0 g/l tryptose, 2.5 g/l lactalbumin hydrolysate and 5% foetal calf serum. Culture supernatants were harvested after full cytopathic effect had developed and virus was concentrated by precipitation with polyethylene glycol (Yamamoto, K.R. et al., *Virology* 40, 734-744, 1970). DNA was released from virus particles by digestion at 37°C for two hours with 100 µg/ml proteinase K (Promega, Wisconsin, USA) in a buffer containing 20mM Tris-HCl (pH 7.5), 10 mM EDTA and 0.5% SDS. After repeated extractions with a 1:1 mixture of phenol/chloroform, nucleic acids were precipitated with two volumes of ethanol and dissolved in TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). Viral DNA was partially digested with the restriction enzyme Sau3A (Promega, Wisconsin, USA) according to the conditions recommended by the enzyme supplier and reaction products were separated on a preparative 0.8% agarose gel.

Fragments of the size fraction between 10 and 15 kb were isolated and ligated 2 hours at 15°C with DNA from bacteriophage lambda EMBL4 digested with BamHI and Sall (Kaiser, K. and Murray, N. in "DNA Cloning", Volume 1, Chapter 1, IRL Press, 1985). Reaction products were packaged in vitro (Promega, Wisconsin, USA) and recombinant phage was plated on E.coli host strain LE392. The library in lambda EMBL4 was enriched for recombinants containing inserts with sequences specifically present in relatively large Sall restriction fragments of the viral genome by screening nitrocellulose replica filters with a ³²P-labelled DNA probe consisting of 10-15 kb restriction fragments isolated by preparative agarose gel electrophoresis of FHV genomic DNA digested with Sall (for technical details see Sambrook, J. et al., in "Molecular Cloning:

A laboratory manual", Chapter 2, Cold Spring Harbor Laboratory Press, 1989). Individual recombinants obtained from this screening procedure were amplified and the restriction pattern of the lambda insert DNA were compared with the published map of the complete FHV genome (Grail, A. et al., *Arch. Virol.*, 116, 209-220, 1991). One of the isolates designated λFHV02, was selected for further study and the 10.9 kb insert of this clone (see Fig. 1) was positioned near the left end of the unique long segment of the viral genome between unit 0.08 and 0.17 on the map of Grail et al., supra.

- Insertion of a marker gene.

The 5.1 kb BamHI fragment of λFHV02 which was subcloned in pGEM3Z resulting in pFHV01 (see Fig. 2A), revealed a unique BglII restriction site in a suitable position for the integration of a marker gene. The gene used for insertion was derived from pCH110 (Pharmacia, Uppsala, Sweden) by replacing a 72 bp SphI fragment near the SV40 origin of replication as present in pCH110 by a double stranded synthetic oligonucleotide with the following structure (SEQ ID No.: 3 and 4):

5'-GGATCCGTCGACCATG-3'

3'-GTACCCTAGGCAGCTG-5'

Insertion of the linker between the two SphI restriction sites of pCH110 does not restore the recognition sequence for SphI on either site and creates both a BamHI and Sall site upstream of the SV40 early promoter. Subsequent digestion with BamHI generated a 4.0 kb

β-galactosidase expression cassette which was inserted at the BglII site of pFHV01 resulting in pFHV04 (see Fig. 2B). Linearized DNA of plasmid pFHV04 was introduced together with viral DNA into CRFK cells by calciumphosphate-mediated DNA precipitation (Graham, F. L. and v.d. Eb, A. J., *Virology* 52, 456-467, 1973). One microgram of DNA from pFHV04 were mixed with 15 microgram of DNA from FHV infected

- cells in a final volume of 376 μ l H₂O and added to 500 μ l of 2x HBSP (10 mM KCl, 280 mM NaCl, 12 mM glucose, 1.5 mM Na₂HPO₄, 50 mM HEPES, pH 7.0). Precipitates were formed by gradually adding 124 μ l of 1 M CaCl₂ solution and incubating the mixtures at room-temperature for 30 minutes. The suspension of precipitated DNA was gently added to two \varnothing 6 cm dishes containing each a semiconfluent monolayer of CRFK cells in 5 ml of culture medium. After 5 hours, medium was removed and 5 ml of HBSP with 15% glycerol was layered onto the cells. After a one to two minute incubation, the solution was removed, cells were washed with medium and dishes were incubated with overlays of 0.75% agarose in culture medium. After 3 to 4 days when cytopathic effect started to develop, a second agarose overlay containing the substrate Bluogal (Gibco-BRL, Maryland, USA) with a final concentration of 0.2 mg/ml, was added and plates were incubated until blue plaques were detected. Positive plaques were picked macroscopically and transferred to flasks with fresh CRFK cells in order to amplify the virus. The plating procedure and plaque isolation was continued until homogeneous stocks of recombinant virus had been established. Virus material from the final preparations was used for detailed analysis of the viral genome by Southern blotting and for animal vaccination experiments.
- 15 Recombinant FHV containing the β -galactosidase marker gene inserted at the BglIII site as present in pFHV01, was shown to be stable upon serial passage in tissue culture on CRFK cells.

- Structural analysis of the insertion region in the unique long segment of the FHV genome.

- 20 The nucleotide sequence analysis was performed on relevant parts of the 5.1 kb BamHI fragment present in pFHV01. The same fragment was thereto subcloned in both orientations into the BglIII site of pSP72 (Promega, Wisconsin, USA) resulting in pFHV02 and pFHV03 respectively.
- Progressive deletions were introduced using the enzyme exonuclease III (Henikoff, S., Gene 28, 351-359, 1984) after double digestion of the plasmid DNA with the appropriate restriction enzymes creating a 5'- and 3'-overhanging extremity. The presence of a 3'-overhanging single strand extremity prevented the plasmid vector DNA from being degraded by exonuclease III. Samples of the reaction mixture were taken at 30 seconds intervals and treated according to Henikoff supra., generating recircularized DNA molecules which were transformed into competent E.coli cells. Plasmid DNA from mini-preparations of individual colonies were analyzed by restriction mapping for the size of the deletion introduced in the original 5.1 kb fragment. Series of candidates containing progressive deletions were analyzed by nucleotide sequencing on double stranded DNA in a chain termination reaction using T7 polymerase (Pharmacia, Uppsala, Sweden). Incomplete or ambiguous readings within the nucleotide sequence were resolved by specific priming of the chain elongation reaction on the inserted DNA of plasmid pFHV01, pFHV02 or pFHV03. Sequence data were assembled and analyzed using Gene-Master (Bio-Rad, California, USA) or equivalent software. Assemblage of all data resulted in an about 1.0 kb region (SEQ ID NO:1) within the unique long segment of the FHV genome consisting of a 579 nucleotide open reading frame encoding a polypeptide with an amino acid sequence shown in SEQ ID NO:2 containing the actual BglIII restriction site used for the insertion of a marker gene and about 0.4 kb of non-translated flanking DNA sequence. The region of about 1.0 kb can be applied for the insertion of foreign genes into the genome of FHV without disabling essential viral functions necessary for infection and replication.

EXAMPLE 2

- 45 Construction of recombinant FHV expressing the envelope protein from feline leukemia virus (FeLV).

- Based on the sequence as presented in SEQ ID:1, a fragment was derived from pFHV02 by trimming both ends of the 5.1 kb BamHI insert using the enzyme exonuclease III and following similar procedures as described for the nucleotide sequence analysis. This resulted in pFHV24 containing a 0.9kb insert in pGEM7Zf(+) (Promega, Wisconsin, USA) with the unique BglIII restriction site previously defined in pFHV01 correctly positioned for the integration of foreign DNA and subsequent in vivo recombination with the viral genome (see Fig. 3A). A strong promoter which could direct the expression of foreign genes after their insertion into the genome of the FHV virus was selected from the LTR sequence of Rous sarcoma virus (RSV).
- 55 The promoter has been mapped on a 580 bp NdeI/HindIII restriction fragment from pRSVcat (Gorman, C. M. et al., Proc. Natl. Acad. Sci. USA 79, 6777-6781, 1982) and was inserted between the HindIII and PstI sites of pGEM3Z by means of double stranded synthetic linkers on both sides of the fragment. The connection between the HindIII site from the vector pGEM3Z and the NdeI site of the RSV fragment

carrying the LTR promotor was made with a 30 bp linker containing cohesive ends compatible with HindIII on one and NdeI on the other site. However, after ligation both restriction sites are not restored due to deliberate modifications in the outer nucleotides of the six basepair recognition sequence. In addition to the removal of these two sites, a new restriction site (BamHI) present within the linker itself was created at the corresponding position. A second 20 bp linker was synthesized which connected the HindIII site from the LTR fragment to the PstI site from pGEM3Z, in this case without destruction of the recognition sequence on either of the ends and adding the convenient unique restriction sites BglII and XhoI, to those already present in the polylinker of pGEM3Z, e.g. PstI, XbaI and BamHI. The resulting derivative of pGEM3Z, designated pVEC01, therefore contains a 650 bp restriction fragment carrying the LTR promotor sequence immediately followed by multiple restriction sites available for the insertion of foreign genes. The 650 bp fragment is flanked on either end by a BamHI restriction site and has been transferred as such to the unique BglII site present in pFHV24. The cohesive ends generated by these two restriction enzymes are compatible but ligation does not restore either of the original recognition sequences for BglII or BamHI. One of the resulting constructs was designated pFHV28 and checked by restriction mapping (Fig. 3B). The structure of this FHV recombination vector allows the insertion of foreign genes immediately downstream the LTR promotor and subsequent integration of the complete expression cassette into the FHV genome by in vivo recombination. The positions of the different restriction sites downstream of the LTR in particular those for the enzymes BglII and XhoI, are designed in such a way that even multiple gene insertion can be envisaged. A first application of this vector consisted of the gene encoding the envelope protein from FeLV/sub-group A and was isolated on a 2.0 kb PstI fragment from pFGA-5 (Stewart, M. A., et al., J. Virol. 58, 825-834, 1986) and subcloned with the coding strand in the T7-orientation of pGEM3Z. The unique SphI site present in the polylinker of the vector was replaced by BamHI through the addition of a synthetic linker similar to the modification introduced in pCH110 (see example 1). The complete gene which could now be isolated on a 2.0 kb BamHI fragment, was inserted at the BglII site of pFHV28 resulting in pFHV29. The correct orientation of the FeLV envelope gene relative to the LTR promotor was confirmed by restriction analysis (see Fig. 4). One microgram of linearized DNA of plasmid pFHV29 was cotransfected with 15 microgram of viral DNA into CRFK cells as described previously in example 1 for the insertion of the β -galactosidase marker gene. The transfection progeny was harvested after 3 to 4 days and seeded in serial dilutions onto CRFK cells in microtiter plates. After cpe had developed, plates were fixed with ethanol and incubated with a rabbit antibody raised against the purified native envelope protein of FeLV. Specifically bound antibodies were detected with a fluorescein-labelled goat anti-rabbit serum and visualized under a UV-microscope. Plaques harbouring recombinant FHV and expressing the envelope protein of FeLV were detected at a frequency of about 5×10^{-4} .

EXAMPLE 3

Construction of recombinant FHV expressing the envelope protein from feline immunodeficiency virus (FIV)

The gene encoding the envelope protein from FIV was also considered as an important candidate to be inserted into the specific region of the U₁ from FHV. The gene was derived from the proviral genome of a Dutch FIV isolate referred to as FIV-UT 113 (Verschoor, E.J. et al., Virology 193, 433-438, 1993) and subcloned as a 2.6 kb BamHI fragment before insertion into the BglII site of pFHV 28 similar to the procedures as described above for the envelope gene of FeLV. The resulting recombination plasmid pFHV 37 (see Fig. 5) was transfected together with FHV viral DNA into CRFK cells as has been described in example 1 for the β -galactosidase expression cassette. The viral progeny of the transfection was seeded on CRFK cells in microtiter plates such that about 50 to 100 pfu per well were obtained. After incubation, the supernatant of each individual well was stored separately and the remainder of the plates was fixed with ethanol. Plates were incubated with the primary reagent consisting of a serum from FIV infected cats. Envelope specific antibodies were detected with a fluorescein-labelled rabbit anti-cat serum and microscopically visualized under UV light.

Wells harbouring positive foci were scored and the corresponding virus samples which had been stored in parallel were seeded again in limiting dilutions of about 1 to 5 pfu per well and treated in a similar way as described above. The plating procedure was repeated until the virus stock appeared to be more than 50% homogeneous for the expression of the envelope protein as detected in an immuno fluorescence assay.

A final purification was performed by single-plaque isolation under an agarose overlay. One of the isolates obtained by this procedure was selected and designated FHV strain F2-1. Amplified stocks with titers of more than 1×10^7 pfu/ml were prepared on CRFK cells and frozen at -80°C until further experimentation.

The viral genome structure of strain F2-1 was analyzed by Southern blotting using a DNA fragment

containing the LTR promotor as a probe in the hybridization. The position of BglII and EcoRI restriction sites in the region of the genome where the envelope gene was inserted was found to be consistent with the expected pattern.

5 EXAMPLE 4

Pathogenicity of FHV mutant in infected animals

10 One of the recombinant viruses constructed as described above in example 1 and containing the β -galactosidase gene inserted at the BglII site present within the 1 kb region of the unique long segment as defined in SEQ ID No.: 1, was designated FHV strain 05-4-1-1. The pathogenicity of this virus and the ability to protect against the clinical signs caused by challenge with a virulent FHV strain was studied in an animal experiment and compared with the parent strain G2620.

15 Specific pathogen-free cats 12 weeks of age were infected oronasally with ca. 1×10^5 TCID₅₀ of the FHV-1 mutant or parent strain by applying 0.3 ml per nostril and 0.4 ml in the oropharynx. Animals were observed daily over a period of 2 weeks for clinical signs specific for FHV-1 infection and scored based on the criteria as listed in Table 1. Cats were challenged six weeks after vaccination by oronasal application of 1×10^5 TCID₅₀ of FHV-1 strain SGE (National Veterinary Service Laboratory, USA) and monitored over a period of 2 weeks for clinical signs of FHV-1 infection. Clinical observations both after vaccination and
20 challenge are summarized in Table 2. Cats in group one that had received an oronasal vaccination with strain 05-4-1-1 showed a reduced level in the score for clinical signs compared to the animals in group two receiving the parent G2620 strain.

After challenge, the vaccinated cats in group 1 and 2 showed an average 10-fold reduction in the clinical scores compared to the non-vaccinated controls in group 3. Therefore it was concluded that the mutant
25 strain 05-4-1-1 had a reduced virulence upon oronasal application in the cat and was still capable of inducing high levels of protection against the clinical signs of a challenge FHV infection.

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Table 1

Clin. sign	Severity	Daily Score (points)
Pyrexia	>39.6 - 39.9	1
	40.0 - 40.4	2
	40.5 - 40.9	3
	>41.0	4
Sneezing	infrequent	1
	frequent	2
	paroxysmal	3
Cough	infrequent	1
	frequent	2
Respiration	URT noise	1
	stertor	1
	mouth breathing	2
Salivation		2
Conjunctivitis	mild	1 per eye
	moderate	2 per eye
	severe	3 per eye
Ocular discharge	serous	1 per eye
	mucopurulent	2 per eye
Nasal discharge	serous	1 per nostril
	mucopurulent	2 per nostril
Ulceration	nasal	2
	nasal/bleeding	3
	oral	2
	oral/bleeding	3
Oral erythema		1
Inappetance		1
Depression		1

Table 2

Group	Vaccine	animal code	clin. scores after vaccination		clin. scores after challenge	
			individual scores	average	individual scores	average
1	05-4-1-1	40L	2	0.5	2	7.3
		40M	0		7	
		40P	0		10	
		40R	0		10	
2	G2620	40V	1	7.0	1	5.5
		40W	8		7	
		40X	5		6	
		H18	14		8	
3	none	H14	-	-	77	79.7
		H12	-		85	
		H11	-		69	
		B39	-		88	

LEGENDS

Figure 1

- 5 Restriction map of the 10.9 kb DNA insert from λ FHV02. The position of this fragment in the viral genome was mapped near the left end of the unique long segment. Subcloning of the 5.1 kb BamHI fragment resulted in pFHV01.

Figure 2

- 10 A - Map of plasmid pFHV01 containing the 5.1 kb BamHI restriction fragment from λ FHV02 subcloned in pGEM3Z.
B - Map of plasmid pFHV04 containing a 4.0 kb β -galactosidase expression cassette inserted at the unique BglII restriction site of pFHV01.

Figure 3

- 15 A - Restriction map of pFHV24. This construct was derived from pFHV02 by eliminating excess sequences flanking the insertion region as defined in SEQ ID:1. The position of the open reading frame (ORF) as defined in SEQ ID:1 is shown at the top.
20 B - Restriction map of plasmid pFHV28, containing the LTR promotor with multiple cloning site inserted at the unique BglII site of pFHV24. This vector enables the integration of foreign genes into the genome of FHV by means of in vivo recombination.

Figure 4

- 25 Restriction map of pFHV29 which contains the gene encoding the envelope protein from FeLV inserted at the BglII site from pFHV28 downstream of the LTR promotor.

Figure 5

- 30 Restriction map of pFHV37 which contains the gene encoding the envelope protein from FIV inserted at the BglII site from pFHV28 downstream of the LTR promotor.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: Akzo N.V.
- (B) STREET: Velperweg 76
- (C) CITY: Arnhem
- (E) COUNTRY: The Netherlands
- (F) POSTAL CODE (ZIP): 6824 BM
- (G) TELEPHONE: 04120-66379
- (H) TELEFAX: 04120-50592
- (I) TELEX: 37503 alpha nl

(ii) TITLE OF INVENTION: Recombinant feline herpesvirus vaccine

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1007 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Feline herpesvirus (FHV-1)
- (B) STRAIN: G 2620

(vii) IMMEDIATE SOURCE:

- (B) CLONE: pFHV01

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 253..834

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

	TA	AC	T	C	T	T	A	T	A	G	T	T	C	G	T	A	A	A	T	T	A	C	T	T	A	T	A	T	T	T	T	T	A		60
5	T	A	A	C	A	G	T	T	A	A	T	T	T	A	C	T	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	120
	T	G	G	T	A	T	T	A	T	A	A	T	C	A	T	T	C	G	T	A	T	A	A	A	A	A	A	A	A	A	A	A	A	A	180
	G	C	G	A	C	G	T	A	A	T	A	C	T	C	T	C	A	C	A	T	T	T	T	A	A	A	A	A	A	A	A	A	A	A	240
10	A	T	T	C	G	C	G	T	G	A	C	A	T	G	C	A	A	G	G	T	A	T	G	A	G	T	C	T	A	C	G	A	T	A	288
15	G	A	G	A	T	A	C	C	A	C	T	G	G	T	A	G	A	T	G	A	A	C	C	A	C	A	C	A	T	C	T	A	T	A	336
20	A	G	G	A	T	T	T	T	A	T	T	T	T	C	T	G	G	T	A	T	G	A	T	T	T	T	T	T	T	T	T	T	T	432	
25	A	T	C	T	A	A	C	A	G	C	T	A	C	T	A	T	T	A	T	A	G	T	A	G	T	A	T	A	T	A	T	A	T	A	480
30	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	576	

EP 0 576 092 A1

CCA AAT CCC CTA AGT GAC TTT ATT ATG CGC GTT AGG CAG ATA TTT TCT 816
Pro Asn Pro Leu Ser Asp Phe Ile Met Arg Val Arg Gln Ile Phe Ser
175 180 185

5 GGA ATC CTA AAT CAT TAATCCATTT ACTAAATAAA TAAACAATAC CGTTTAGGTA 871
Gly Ile Leu Asn His
190

ATTAAACATG ATTCTAGTGT TTATTGTCGT ATGTACGGGC GATGGTTGGA TAACAACTCG 931

10 ACAATGATCA ATTATATTGA TTAACCTTGT AATAAATTCG TCGGATTATT GGATATATCG 991

AGATGATATC ACATTA 1007

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 193 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEO ID NO:2:

Met Gln Gly Met Arg Ser Thr Ser Gln Pro Leu Val Glu Ile Pro Leu
1 5 10 15

Val Asp Met Glu Pro Gln Pro Ser Ile His Ser Asn Glu Pro Asn Pro
20 25 30

Pro Asn Lys Met Leu Thr Thr Ala Ile Ser Ser Arg Arg Ser Gly Ile
35 40 45

Phe Leu Phe Ser Leu Gly Met Phe Phe Phe Gly Val Ile Leu Thr Ala
50 55 60

Thr Ile Ile Val Cys Thr Phe Ile Phe Thr Ile Pro Val Asp Met Leu
65 70 75 80

Gln Met Pro Arg Cys Pro Glu Glu Thr Val Gly Ile Lys Asn Cys Cys
85 90 95

Ile Arg Pro Ile Arg Arg His Val Lys Ser His Gln Asp Leu Val Ala
100 105 110

Thr Cys Ala Glu Tyr Met Glu Gln Pro Ala Thr Ala Ser Ala Val Gly
115 120 125

Ala Leu Ile Pro Leu Leu Asp Ile Phe Asn Gly Asp Gly Ile Ser Thr
130 135 140

Asn Asp Ser Leu Tyr Asp Cys Ile Leu Ser Asp Glu Lys Lys Ser Cys
145 150 155 160

Asn Thr Ser Met Ala Val Cys Gln Ser Thr Tyr Leu Pro Asn Pro Leu
 165 170 175
 Ser Asp Phe Ile Met Arg Val Arg Gln Ile Phe Ser Gly Ile Leu Asn
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His

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 16 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: -
 (B) LOCATION: 1..16
 (D) OTHER INFORMATION: /label= linker_1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGATCCGTCG ACCATG

16

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 16 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: -
 (B) LOCATION: 1..16
 (D) OTHER INFORMATION: /label= linker_2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GTACCCTAGG CAGCTG

16

Claims

1. FHV mutant comprising a mutation in the FHV genome in a region defined by the DNA sequence of an open reading frame encoding a polypeptide shown in SEQ ID NO: 2 or a variant thereof, and flanking intergenic sequences thereof.
2. FHV mutant according to claim 1, characterized in that it comprises a heterologous nucleic acid sequence, said nucleic acid sequence being introduced in an insertion-region of the FHV genome defined by the DNA sequence of an open reading frame encoding a polypeptide shown in SEQ ID NO: 2 and flanking intergenic sequences.
3. FHV mutant according to claim 1 or 2, characterized in that the insertion-region has the DNA sequence shown in SEQ ID NO: 1.

4. FHV mutant according to claims 1-3, characterized in that the heterologous nucleic acid sequence is introduced in the insertion-region defined by the DNA sequence of the open reading frame encoding the polypeptide shown in SEQ ID NO: 2.
5. FHV mutant according to claims 1-4, characterized in that the heterologous nucleic acid sequence is introduced at the BglII restriction site in the open reading frame.
6. FHV mutant according to claims 1-5, characterized in that at least a part of the FHV DNA sequence within the insertion-region is deleted.
7. FHV mutant according to claims 1-6, characterized in that the heterologous nucleic acid sequence encodes a polypeptide and is under control of a promotor regulating the expression of said nucleic acid sequence in a cell infected with said recombinant FHV.
8. FHV mutant according to claims 1-7, characterized in that the heterologous nucleic acid sequence encodes an antigen of a feline pathogen.
9. FHV mutant according to claim 8, characterized in that the antigen is derived from a pathogen selected from the group consisting of feline leukemia virus, feline immunodeficiency virus, feline calicivirus, feline parvovirus, feline coronavirus and feline Chlamydia.
10. Nucleic acid sequence comprising a region of the FHV genome defined by the DNA sequence of an open reading frame encoding a polypeptide shown in SEQ ID NO: 2 or a variant thereof, and flanking intergenic sequences.
11. Nucleic acid sequence comprising a gene heterologous to FHV under control of a promotor, flanked by DNA sequences derived from the insertion-region of the FHV genome defined by the DNA sequence of an open reading frame encoding a polypeptide shown in SEQ ID NO: 2 and flanking intergenic sequences.
12. Recombinant DNA molecule comprising a nucleic acid sequence according to claim 10 or 11.
13. Host cell transfected with a recombinant DNA molecule according to claim 12.
14. Process for the preparation of an FHV mutant according to claims 1-9, characterized in that a cell culture is transfected with FHV genomic DNA and a recombinant DNA molecule according to claim 12.
15. Cell culture infected with an FHV mutant according to claims 1-9.
16. Vaccine comprising an FHV mutant according to claims 1-9.
17. Method for the immunization of animals against an infectious disease which comprises administering a vaccine according to claim 16.

Fig 1

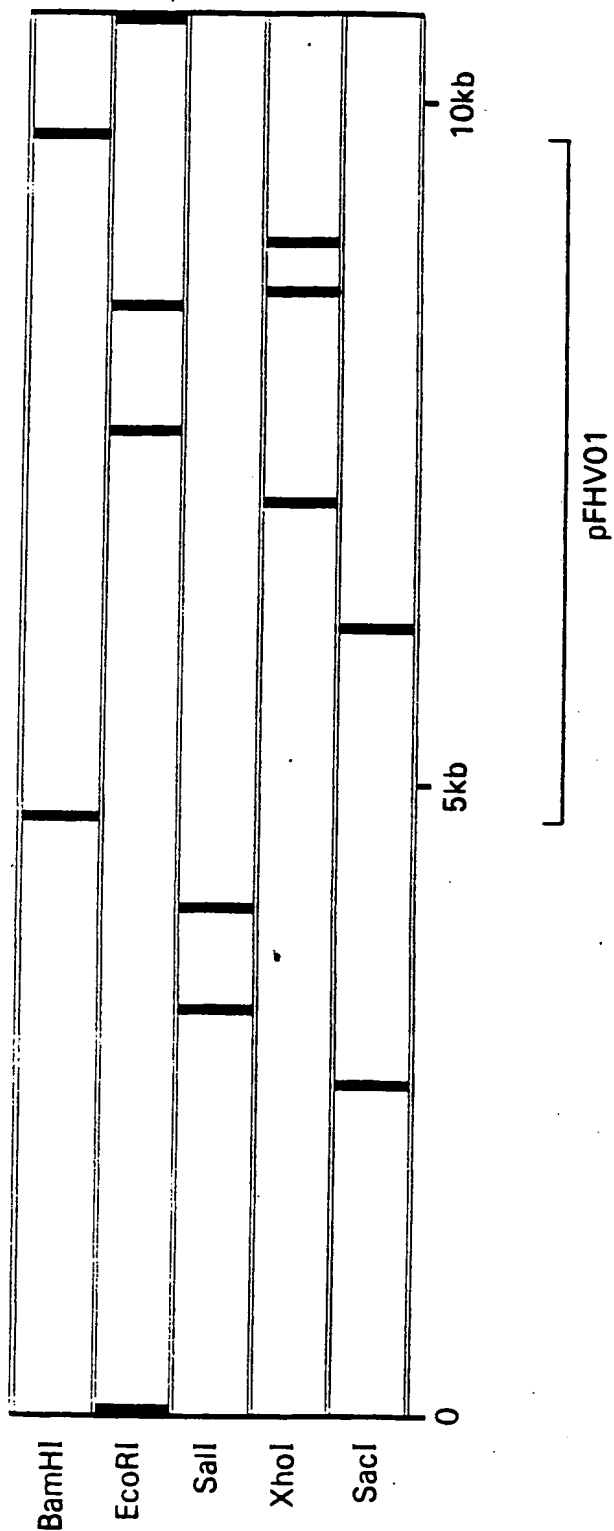


Fig 2

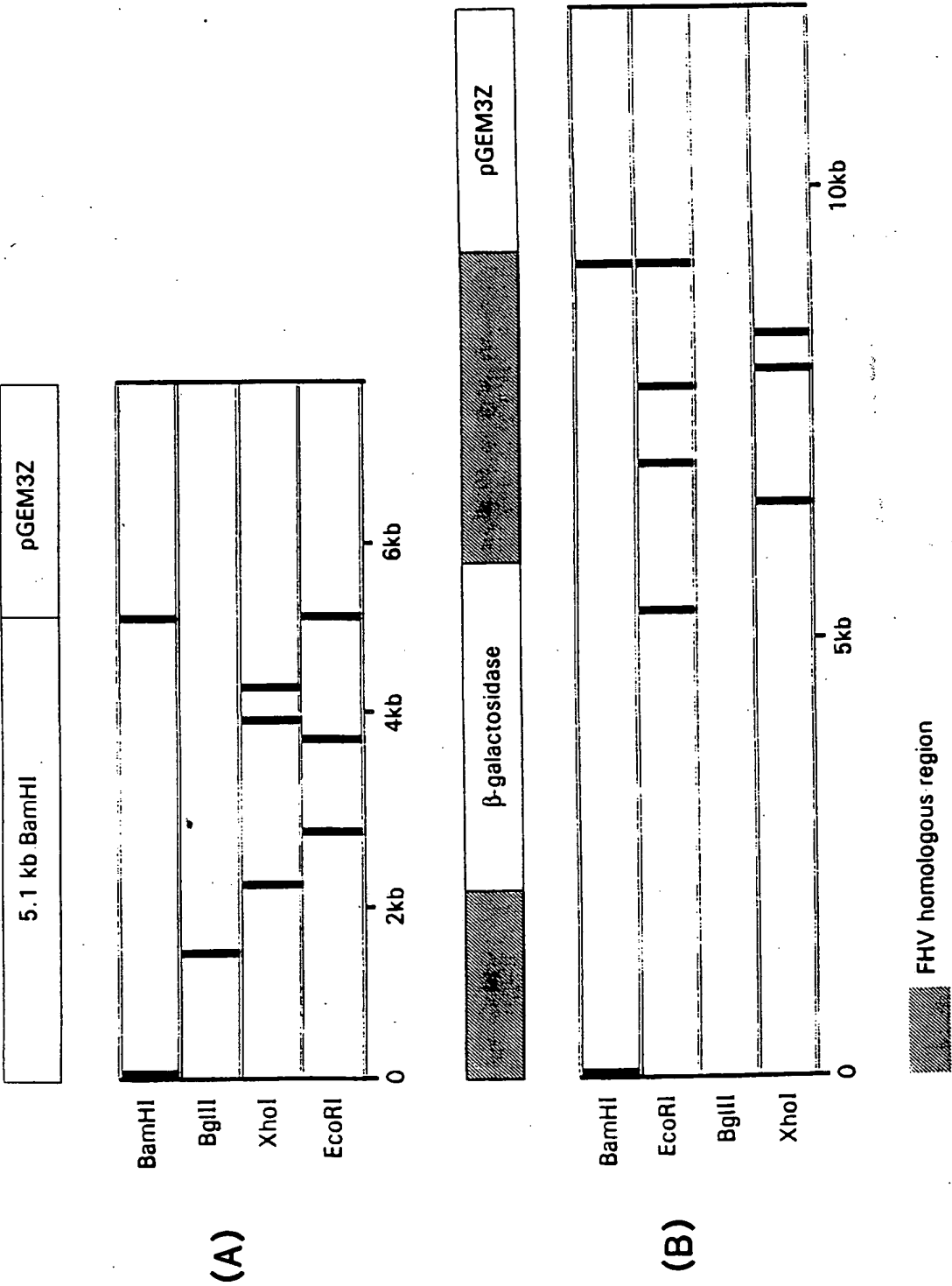


Fig 3

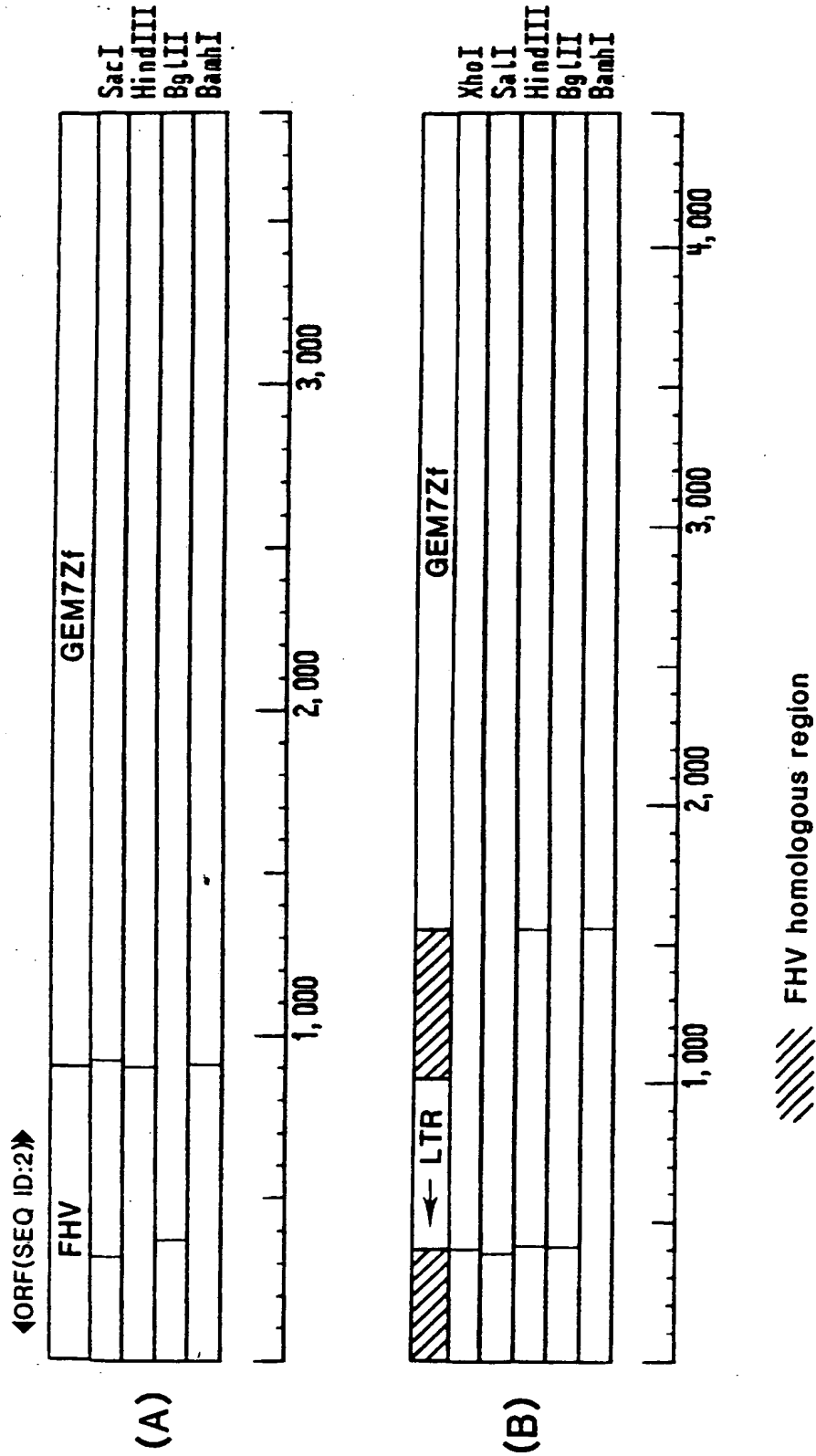


Fig 4

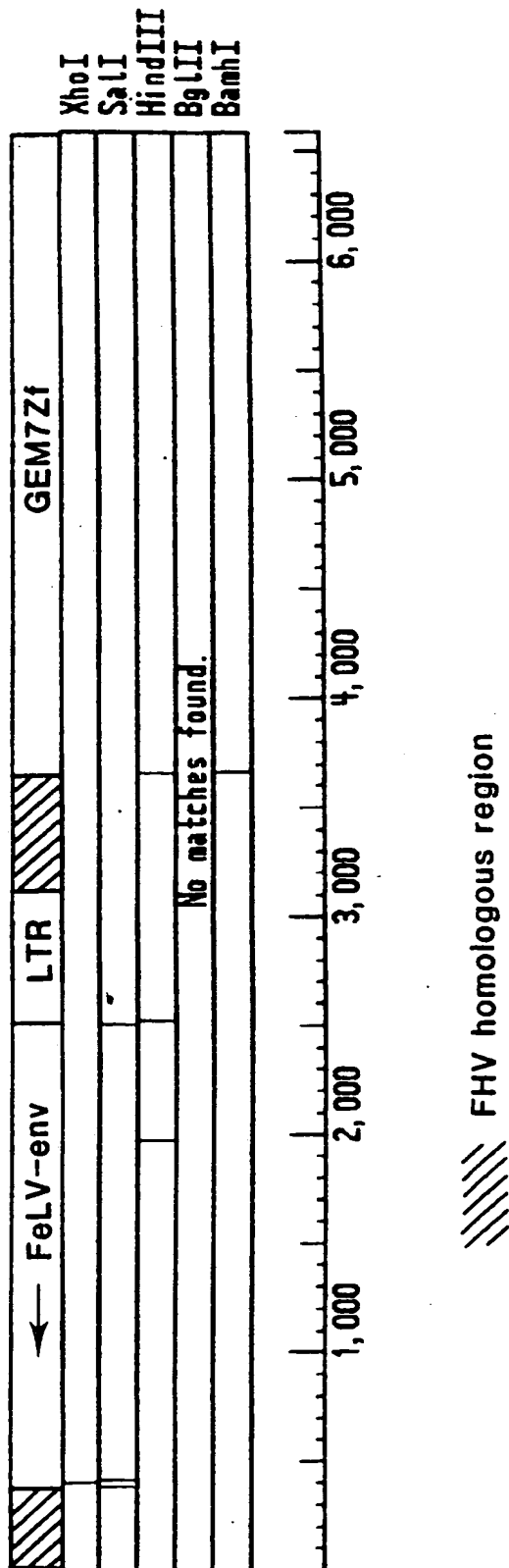
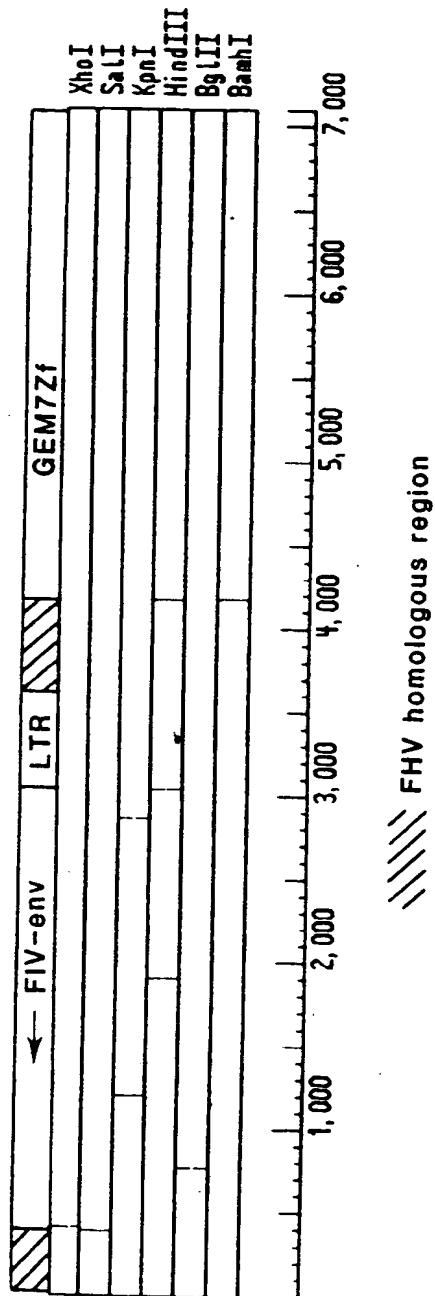


Fig 5





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Application Number

EP 93 20 1791

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DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
Y	WO-A-8 704 463 (SYNTRO CORPORATION) * the whole document * ---	1-17	C12N15/86 C12N15/38 A61K39/245 C07K15/00
D,Y	VIROLOGY vol. 154, no. 1, 15 October 1986, NEW YORK, US pages 168 - 179 P.A. ROTA ET AL. 'Physical characterization of the genome of feline herpesvirus-1' * the whole document * ---	1-17	
A	WO-A-9 001 547 (THE UPJOHN COMPANY ET AL.) * the whole document * ---	1-17	
D,A	JOURNAL OF VIROLOGY vol. 64, no. 10, October 1990, US pages 4930 - 4938 G.E. COLE ET AL. 'Recombinant feline herpesviruses expressing feline leukemia virus envelope and gag proteins' * the whole document * ---	1,7-9, 14-17	
A	VIROLOGY vol. 179, no. 1, November 1990, NEW YORK, US pages 378 - 387 L. NICOLSON ET AL. 'The nucleotide sequence of the equine herpesvirus 4 gC gene homologue' * the whole document * --- -/--	10	
The present search report has been drawn up for all claims			
Place of search BERLIN		Date of completion of the search 03 AUGUST 1993	Examiner JULIA P.
CATE G RY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ----- A : member of the same patent family, corresponding document	

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PAGE2

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A	JOURNAL OF VIROLOGY vol. 62, no. 8, August 1988, US pages 2850 - 2858 G.P. ALLEN ET AL. 'Characterization of an equine herpesvirus type 1 gene encoding a glycoprotein (gp13) with homology to HSV glycoprotein c' * the whole document *	10	
			TECHNICAL FIELDS SEARCHED (Int. Cl.5)
The present search report has been drawn up for all claims			
Place of search BERLIN		Date of completion of the search 03 AUGUST 1993	Examiner JULIA P.
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document			
T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons * : member of the same patent family, corresponding document			

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